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CNR1 gene deletion affects the density of endomorphin-2 binding sites in the mouse brain in a hemisphere-specific manner

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ABSTRACT

Endomorphin-1 (EM-1) and endomorphin-2 (EM-2) are two endogenous tetrapeptides with very high affinities for the μ-opioid receptor. Until recently, the precise neuroanatomical localization of the binding sites for these peptides was unknown. However, the recent synthesis of tritiated forms of these molecules has permitted these binding sites to be analysed with a very high degree of neuroanatomical specificity. Preliminary studies demonstrated a superior binding profile for EM-2, with less non-specific binding than EM-1 . As the endogenous cannabinoid and opioid systems interact at several levels, we investigated how deletion of the *CNR1* gene, which encodes the cannabinoid receptor 1 (CB_1R) protein, affects the brain distribution of EM-2 binding sites. Our results revealed no differences in the average density of EM-2 binding sites in CB_1 receptor knockout (CB_1R KO) and WT mice. However, when both hemispheres were analysed separately, we detected specific alterations in the distribution of EM-2 binding sites in the right hemisphere of CB_1R KO mice. While, the density of EM-2 binding sites in CB_1R KO mice was higher in the CA3 hippocampal field and in the pontine tegmental nuclei, it was lower in the superior colliculus and ventral tegmental area than in WT controls. No differences were observed in the left hemisphere for any of the regions analysed. For the first time these findings demonstrate a lateralization effect on cerebral opioid binding sites that may be mediated by the central cannabinoid system.

1. Introduction

Endomorphin-1 (Tyr–Pro–Trp–Phe–NH_2; EM-1) and endomorphin-2 (Tyr–Pro–Phe–Phe–NH_2; EM-2) are two endogenous neuropeptides that act as natural opioid ligands for the μ-opioid receptor in mammals, including humans (Zadina et al., 1999). The selectivity of these ligands for μ-opioid receptors has been demonstrated in several autoradiographic (Goldberg et al., 1998; Kakizawa et al., 1998; Sim et al., 1998) and in vitro studies (Gong et al., 1998). Endomorphins modulate the physiological effects of opioids by activating μ-opioid receptors. For example, intracerebroventricular (i.c.v.) administration of endomorphins induces a potent antinociceptive effect in wild type mice (Horvath, 2000) but not in μ-opioid receptor knockout mice (Loh et al., 1998).

The functional bidirectional cooperation between the cannabinoid and opioid systems is well documented, and these systems share many pharmacological properties and roles in: antinociception, tolerance-dependence phenomena, hypothermia, emesis (Simoneau II et al., 2001), sedation/catalepsy and intestinal motility (Basilico et al., 1999; Frederickson et al., 1976; Izzo et al., 1999), immune reactivity (Massi et al., 2001), and emotional and locomotor behaviour (Berrendero and Maldonado, 2002), among others. This functional interaction may occur at the level of the receptor and its signal transduction pathways, cannabinoids inducing the release of opioid peptides, or opioids promoting endocannabinoid release. Both CB_1 and μ-opioid receptors are predominantly located presynaptically, where they inhibit the release of different neurotransmitters (Mansour et al., 1995; Schlicker and Kathmann, 2001) by modulating intracellular pathways that respond to the activation of Gi/Go GTP-binding proteins (Connor and Christie, 1999; Howlett et al., 2004).

Deletion of the CB_1 receptor gene alters the expression of μ-opioid receptors and dynorphin and enkephalin peptides in

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several brain areas (Lane et al., 2010; Steiner et al., 1999; Zimmer et al., 1999), explaining the physiological alterations in opioid transmission seen in the CB_1R KO mice. However, the binding potential and expression of μ-opioid receptors has yet to be studied in these mice. We analysed the distribution of EM-2 binding sites in male mice with a genetic deletion of the CB_1 receptor gene using a relatively new tritiated radioligand instead of the ^{125}I ligands usually used in autoradiography studies. Tritiation of EM-2 by dehalotritiation produces specific labelling (Monory et al., 2000) with a long half-life and low radiant energy, making it more convenient to handle than ^{125}I. Here, we focused on the distribution of EM-2 rather than EM-1, given its superior profile and reduced non-specific binding.

2. Materials and methods

2.1. Animals

Adult male CD1 wild-type ($CB_1R^{+/+}$; $n=4$) and transgenic homozygous CB_1R knockout ($CB_1R^{-/-}$; $n=4$) mice weighing approximately 30 g at the beginning of the experiments, were used in this study. These mice were generated as previously described (Ledent et al., 1999). Animals were housed in conditions of controlled temperature (21 ± 2 °C) and light (on 07:00 h, off 19:00 h), with ad libitum access to water and food. All handling and experimental procedures were conducted in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian legislation for the Protection of Animals in Research (XXVIII.tv. Section 32.)

2.2. Synthesis of tritiated endomorphin 2

3′,5′-^{3}H-Tyr ^{1}-endomorhin 2 (Tyr–Pro–Phe-Phe–NH_2: [^{3}H]–EM-2) was synthesized as described previously (Monory et al., 2000). Briefly, [3H]–EM-2 was prepared by catalytic dehalogenation of a diionated precursor ([3′-5′-I]Tyr–Pro–Phe–Phe–NH_2) using a $Pd/BaSO_4$ catalyst and $^3H_2$ gas in a tritium manifold (Tömböly et al., 2001). The crude tritiated peptide was purified by thin layer chromatography on a Kieselgel 60F254 plate (BuOH: AcOH:H_2O, 4:1:1) or by high performance liquid chromatography (HPLC). Purity was determined by HPLC (LiChrospherR 100 RP-18 5 mm, Rt, 10.51 min: Merck, Darmstadt, Germany) using acetonitrile/0.1% TFA in water as the eluent in a gradient of 20–35 % organic component over 25 min (flow rate, 1 mL/min) and it was found to be > 95%. The specific radioactivity was 1.98 TBq/mmol (39.24 Ci/mmol).

2.3. Quantitative receptor autoradiography

Mice were decapitated, and their brain was quickly removed and frozen at −80 °C. Coronal brain sections were obtained at the level of the NAcc, thalamus, ventral tegmental area (VTA) and locus coeruleus (LC), according to the mouse brain atlas of Franklin and Paxinos (Franklin and Paxinos, 2007), and they were mounted on gelatin-coated slides and stored at −80 °C until the day of the assay. EM-2 binding at brain sites was analysed using the method described by Mansour et al. (1987) to study μ-opioid receptors, with some modifications. Briefly, brain sections were preincubated for 6 min in Tris–HCl buffer (100 mM, pH 7.4) and then for 1 h at 4 °C with 3 nM [3 H]–EM-2 in Tris–HCl buffer (50 mM, pH 7.4), either in the presence or absence of 10 μM unlabelled EM-2 to determine non-specific and total binding, respectively. Subsequently, the slides were washed twice (2 × 6 min) in cold Tris–HCl buffer (50 mM, pH 7.4), rinsed briefly in the same buffer and washed twice in distilled water. The sections were then dried under a stream of cool air and they were then apposed to tritium-sensitive film ([[3H]]-Hyperfilm, Amersham, United Kingdom) in standard X-ray cassettes and exposed for a period of 16 weeks at 4 °C. At the end of this exposure period the films were developed for 5 min at -20 °C in a Kodak D-19 developer, fixed for 10 min, and finally rinsed in water and air-dried . Autoradiograms were analysed using the Scion Image software (Scion Corp. Frederick MD, USA). Density measurements were taken from each hemisphere in 2–4 consecutive brain sections per animal and they were transformed to concentrations (nCi/mg tissue) using tritium-labelled microscale standards (Amersham). Averaged levels were obtained by calculating the arithmetic mean of the measurements taken in both hemispheres.

2.4. Statistical analysis.

The data were expressed as the mean ± S.E.M and analysed using a two-tailed Student *t*-test for independent samples. The significance level was set at ($\alpha=0.05$. All analyses were performed with IBM Statistics version 19.

3. Results

Specific EM-2 binding was analysed in several brain regions of WT and CB_1R KO mice. A heterogeneous binding pattern was observed throughout the brain with several structures exhibiting high levels of binding, including the striatum (Caudate-Putamen – CPu- and NAcc), limbic areas (such as the amygdala) and other nuclei of the pons (e.g., the tegmental nuclei (NTeg), and the superior and inferior colliculi). No differences were found between WT and CB_1R KO mice when the levels of binding were averaged across the hemispheres. However, when separate analyses were performed for each hemisphere, CB_1R KO mice exhibited denser EM-2 binding in the CA3 region of the hippocampus (Tables 1 and 2 and Figs. 1 and 2) and in the NTeg (mostly the laterodorsal tegmental nucleus) of the right hemisphere, whereas no such differences were observed in the left hemisphere (Table 4 and Fig. 4). By contrast, CB_1R KO mice exhibited fewer EM-2 binding sites in the superior colliculus (SupCol) and the VTA of the right hemisphere when compared with WT controls (Table 3 and Fig. 3).

4. Discussion

This study describes the effects of genetic deletion of the *CNR1* gene on the density of EM-2 binding sites in the mouse brain. When analysed globally, comparable levels of EM-2 binding were observed in WT and CB_1R KO mice. However, hemisphere-specific analyses revealed an interesting effect in the right hemisphere, where EM-2 binding increased in the CA3 and NTeg of CB_1R KO mice, with less binding in the VTA and ColSup. No such effects were observed in the left hemisphere. These results demonstrate

Table 1
Table show the *t* statistic and corresponding *p* value for EM-2 binding in each hemisphere and the average values for both hemispheres combined.

Nucleus	Total	Right hemisphere	Left hemisphere
CPu	$t_6=0.800$, $P=$n.s.	$t_6=0.811$, $P=$n.s.	$t_6=0.731$, $P=$n.s.
Shell	$t_6=0.352$, $P=$n.s.	$t_6=1.267$, $P=$n.s.	$t_6=0.051$, $P=$n.s.
Core	$t_6=0.526$, $P=$n.s.	$t_6=0.081$, $P=$n.s.	$t_6=0.901$, $P=$n.s.
Cing	$t_6=0.594$, $P=$n.s.	$t_6=0.230$, $P=$n.s.	$t_6=0.973$, $P=$n.s.
Enthor	$t_6=0.320$, $P=$n.s.	$t_6=0.468$, $P=$n.s.	$t_6=0.112$, $P=$n.s.
Matrix	$t_6=0.793$, $P=$n.s.	$t_6=0.980$, $P=$n.s.	$t_6=0.068$, $P=$n.s.
Striosomes	$t_6=0.684$, $P=$n.s.	$t_6=0.759$, $P=$ns.	$t_6=0.518$, $P=$n.s.